

Roles of 1-Phosphatidylinositol 3-Kinase and ras in Regulating Translocation of GLUT4 in Transfected Rat Adipose Cells

MICHAEL J. QUON,¹ HUI CHEN,¹ BRIAN L. ING,² MIAN-LAI LIU,² MARY JANE ZARNOWSKI,²
KAZUYOSHI YONEZAWA,³ MASATO KASUGA,³ SAMUEL W. CUSHMAN,²
AND SIMEON I. TAYLOR^{2*}

Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases,² and Hypertension-Endocrine Branch, National Heart Lung and Blood Institute,¹ National Institutes of Health, Bethesda, Maryland 20892, and Second Department of Internal Medicine, Kobe University School of Medicine, Chuo-ku, Kobe 650, Japan³

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Insulin stimulates glucose transport in insulin target tissues by recruiting glucose transporters (primarily GLUT4) from an intracellular compartment to the cell surface. Previous studies have demonstrated that insulin receptor tyrosine kinase activity and subsequent phosphorylation of insulin receptor substrate 1 (IRS-1) contribute to mediating the effect of insulin on glucose transport. We have now investigated the roles of 1-phosphatidylinositol 3-kinase (PI 3-kinase) and ras, two signaling proteins located downstream from tyrosine phosphorylation. Rat adipose cells were cotransfected with expression vectors that allowed transient expression of epitope-tagged GLUT4 and the other genes of interest. Overexpression of a mutant p85 regulatory subunit of PI 3-kinase lacking the ability to bind and activate the p110 catalytic subunit exerted a dominant negative effect to inhibit insulin-stimulated translocation of epitope-tagged GLUT4 to the cell surface. In addition, treatment of control cells with wortmannin (an inhibitor of PI 3-kinase) abolished the ability of insulin to recruit epitope-tagged GLUT4 to the cell surface. Thus, our data suggest that PI 3-kinase plays an essential role in insulin-stimulated GLUT4 recruitment in insulin target tissues. In contrast, overexpression of a constitutively active mutant of ras (L61-ras) resulted in high levels of cell surface GLUT4 in the absence of insulin that were comparable to levels seen in control cells treated with a maximally stimulating dose of insulin. However, wortmannin treatment of cells overexpressing L61-ras resulted in only a small decrease in the amount of cell surface GLUT4 compared with that of the same cells in the absence of wortmannin. Therefore, while activated ras is sufficient to recruit GLUT4 to the cell surface, it does so by a different mechanism that is probably not involved in the mechanism by which insulin stimulates GLUT4 translocation in physiological target tissues.

The biological actions of insulin are important for regulation of growth, differentiation, and metabolism. One of the most important metabolic actions of insulin is to stimulate glucose transport in tissues such as muscle and fat. In these classical insulin target tissues, the ability of insulin to recruit glucose transporters (primarily GLUT4) to the cell surface in a dose-dependent reversible manner accounts for the majority, if not all, of the increase in glucose uptake seen with insulin stimulation (8, 26, 35, 40). Recently, tremendous progress in understanding growth factor signaling has led to major advances in understanding the molecular mechanisms of insulin signaling (for reviews, see references 29 and 46). However, the signaling mechanisms involved in insulin-stimulated glucose transport and GLUT4 translocation are not well understood.

Using a transient-transfection system for rat adipose cells in primary culture (32), we previously demonstrated roles for the insulin receptor tyrosine kinase and insulin receptor substrate 1 (IRS-1) in insulin signaling pathways related to GLUT4 translocation in a physiologically relevant tissue (30, 31). We now apply our transfection system to investigate the roles of 1-phosphatidylinositol 3-kinase (PI 3-kinase) and ras, two signaling proteins located downstream of IRS-1 and other substrates of the insulin receptor. When IRS-1 and other insulin

receptor substrates such as Shc and IRS-2 are phosphorylated by the insulin receptor, phosphotyrosine motifs on these substrates interact directly with src homology 2 (SH2) domain-containing proteins, such as PI 3-kinase, GRB-2, and SHPTP-2 (22, 29). The adaptor molecule GRB-2 is preassociated with Sos, a guanine nucleotide exchange factor that promotes the formation of the active GTP-bound state of ras (for a review, see references 29 and 46). Although PI 3-kinase and ras appear to be on separate signaling pathways branching from IRS-1, both have been implicated in pathways that mediate the mitogenic actions of insulin (12, 18, 20, 24, 34, 43).

PI 3-kinase is a heterodimer consisting of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (24). Phosphorylated YMXM motifs on IRS-1 directly interact with the SH2 domains of p85, resulting in activation of p110 (5, 43, 50). Expression of a dominant negative mutant of p85 (Δ p85) inhibited the ability of insulin to stimulate recruitment of glucose transporters in CHO cells (9). However, the principal glucose transporter in CHO cells is GLUT1. Thus, we have carried out similar studies in the physiologically relevant rat adipose cell, whose principal insulin-responsive glucose transporter is GLUT4. Expression of Δ p85 in adipose cells results in inhibition of the ability of insulin to recruit cotransfected epitope-tagged GLUT4 to the cell surface. In addition, treatment with wortmannin (an inhibitor of PI 3-kinase) abolishes insulin-stimulated translocation of epitope-tagged GLUT4 in the control cells. These results are consistent with previous

* Corresponding author. Mailing address: NIH, NIDDK, Diabetes Branch, Building 10, Room 8S-235A, 10 Center Dr. MSC 1770, Bethesda, MD 20892-1770. Phone: (301) 496-4658. Fax: (301) 402-0573.

data showing that inhibitors of PI 3-kinase can impair insulin-stimulated glucose transport in rat adipose cells (23) and 3T3-L1 adipocytes (3, 6). Our results provide direct evidence for an essential role for the p85 regulatory subunit of PI 3-kinase in insulin-stimulated GLUT4 translocation in the physiologically relevant adipose cell.

Recent studies in transfected 3T3-L1 adipocytes and cardiac myocytes have argued both for (14, 19) and against (10, 44) a role for ras in the acute response of insulin to stimulate glucose transport and GLUT4 translocation. We have examined the effects of transfecting adipose cells with constitutively active and dominant negative mutants of ras. Our results suggest that constitutively active ras is sufficient to recruit GLUT4 to the cell surface in the absence of insulin, while dominant negative ras has no significant effect on GLUT4 translocation. However, wortmannin treatment of cells overexpressing constitutively active ras causes only a small decrease in the amount of GLUT4 recruited to the cell surface by activated ras. Therefore, unlike PI 3-kinase, ras is probably not involved in the physiological insulin signaling pathway leading to GLUT4 recruitment in insulin target tissues.

MATERIALS AND METHODS

DNA vector constructions. pCIS2 is an expression vector containing a cytomegalovirus promoter and enhancer with a generic intron located upstream from the multiple cloning site (obtained from Cornelia Gorman, Genentech) (4). This vector, which has been shown to give high expression in transfected rat adipose cells (32), was used as the parent vector for some of the subsequent constructions.

GLUT4-HA, the pCIS2 vector containing the cDNA coding for human GLUT4 with the influenza virus hemagglutinin epitope (HA1) inserted in the first exofacial loop of GLUT4, was constructed as described before (31).

The SR α expression vector (obtained from Richard Roth, Stanford University), which has been shown to give good expression in transfected rat adipose cells (32), was used as the parent vector for the SR α -Wp85 and SR α - Δ p85 constructions.

For SR α -Wp85, the entire coding sequence of bovine p85 α was subcloned into the SR α plasmid as described before (9).

For SR α - Δ p85, a deletion mutant of bovine p85 α that lacks a binding site for the p110 catalytic subunit of PI 3-kinase was constructed and subcloned into SR α as described before (9). The mutant p85 α has a deletion of 35 amino acids (residues 479 to 513) and the insertion of two amino acids (Ser-Arg) replacing the deleted sequence.

For pCIS-L61-ras, a *Bam*HI fragment containing the cDNA for human ras^H with an activating mutation substituting leucine for glutamine at position 61 (obtained from D. R. Lowy [47]) was blunt ended and ligated into the *Hpa*I site of pCIS2 in the sense orientation.

For pCIS-v-ras, a *Sac*I-*Xho*I fragment containing the cDNA for human ras^H with activating mutations substituting arginine for glycine at position 12 and threonine for alanine at position 59 (obtained from D. R. Lowy [47]) was blunt ended and ligated into the *Hpa*I site of pCIS2 in the sense orientation.

For pCIS-N17-ras, a *Sal*I-*Xho*I fragment containing the cDNA for human ras^H with a dominant negative mutation substituting asparagine for serine at position 17 (obtained from L. Feig and T. Finkel) was blunt ended and ligated into the *Hpa*I site of pCIS2 in the sense orientation.

SRE-luc is a plasmid containing the serum response element of *c-fos* inserted upstream of the herpes simplex virus thymidine kinase minimal promoter driving expression of the luciferase reporter gene and was used to assess the function of the transfected ras constructs (obtained from J. E. Pessin) (48).

Milligram quantities of the plasmid DNA vectors described above were obtained with a Magic Megaprep kit (Promega). The concentration of plasmid DNA was determined by comparison with known DNA markers by ethidium bromide staining of restriction-digested plasmids run on an agarose gel.

Isolated rat adipose cell preparation. Isolated adipose cells were prepared from the epididymal fat pads of male rats (170 to 200 g, CD strain; Charles River Breeding Laboratories, Wilmington, Mass.) by collagenase digestion as described before (13, 31, 32).

Electroporation. Isolated adipose cells were transfected by electroporation as described before (31, 32). Cells from multiple cuvettes were pooled to obtain the necessary volume of cells for each experiment. Table 1 shows the combinations and concentrations of plasmid DNA as well as the number of cuvettes used for the insulin dose-response experiments involving transfection of SR α - Δ p85, pCIS-L61-ras, pCIS-v-ras, and pCIS-N17-ras. Table 2 shows the combinations and concentrations of plasmid DNA used for the experiments involving transfection of SR α -Wp85.

TABLE 1. Transfection of SR α - Δ p85 and ras constructs into rat adipose cells^a

Group	No. of cuvettes	GLUT4-HA (μ g/cuvette)	Experimental (μ g/cuvette)	pCIS2 (μ g/cuvette)
Experimental	30	3	6	—
Control	30	3	—	6
Nonspecific	10	—	—	9

^a The amount of plasmid DNA and the number of cuvettes used for each of the transfection experiments are shown. The experimental group cotransfected with GLUT4-HA and either SR α - Δ p85, pCIS-L61-ras, pCIS-v-ras, or pCIS-N17-ras was compared with the control group cotransfected with pCIS2 and GLUT4-HA. A group transfected with pCIS2 alone was used in each experiment to determine the nonspecific signal. Each individual experiment was performed on pools of cells obtained by combining the contents of the cuvettes from each group. All experiments were repeated at least four times. All cells were exposed to a total DNA concentration of 9 μ g per cuvette.

Assay for cell surface epitope-tagged GLUT4. At 20 h after electroporation, adipose cells were processed as described before (30–32) and treated with insulin at final concentrations of 0, 0.024, 0.072, 0.3, and 60 nM at 37°C for 30 min. For experiments that used wortmannin, electroporated cells were incubated in the presence or absence of 500 nM wortmannin (20-h and 30-min incubations gave similar results) before being processed and treated or not with 60 nM insulin for 30 min. Cell surface epitope-tagged GLUT4 was determined by using the anti-HA1 mouse monoclonal antibody 12CA5 (Boehringer-Mannheim, Indianapolis, Ind.) in conjunction with sheep anti-mouse ¹²⁵I-immunoglobulin G (IgG) as described before (30–32). Cells transfected with the parent vector (either pCIS2 or SR α) were used to determine nonspecific binding of the antibodies. Typically, the nonspecific binding was ~30% of the total binding to cells transfected with GLUT4-HA and maximally stimulated with insulin (31). The actual specific counts were comparable from experiment to experiment (see figure legends). The lipid weight from a 200- μ l aliquot of cells was determined as described before (7) and used to normalize the data for each sample.

Cotransfection of ras constructs and SRE-luc. Adipose cells were cotransfected with SRE-luc (1 μ g per cuvette) and either pCIS2, pCIS-L61-ras, pCIS-v-ras, or pCIS-N17-ras (6 μ g per cuvette). At 20 h after electroporation, the cells were processed as described before (30–32) and incubated with or without insulin at a final concentration of 60 nM at 37°C for 6 h. Luciferase activity was determined for each sample as described before (32) and normalized by using the lipid weight from a 200- μ l aliquot of cells.

Immunoprecipitation and immunoblotting of GLUT4-HA. Total membrane fractions were prepared from adipose cells cotransfected with GLUT4-HA (3 μ g per cuvette) and either pCIS, SR α - Δ p85, pCIS-L61, pCIS-v-ras, or pCIS-N17-ras (6 μ g per cuvette) as follows. Pooled cells (20 cuvettes for each group) were washed once and then resuspended in an equal volume of TES (20 mM Tris, 1 mM EDTA, 8.73% sucrose, 1 mM phenylmethylsulfonyl fluoride [pH 7.4]) at 18°C. Cells were homogenized by passage three times through a 25-gauge needle. The homogenates were then centrifuged for 10 min at 400 \times g, 4°C. The pellet and fat cake were discarded, and the infranant was centrifuged for 30 min at 400,000 \times g, 4°C. The resulting pellet (containing the total membrane fraction) was resuspended in 500 μ l of TES containing a cocktail of protease inhibitors (10 mg of bovine serum albumin, 10 μ g of leupeptin, and 10 μ g of aprotinin per ml) and stored at -80°C until further processing within 24 h. After thawing, samples were centrifuged at 200,000 \times g, 4°C, for 90 min, and the pellets were resuspended in 50 μ l of 150 mM NaCl–50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4) (buffer A) containing 1% Triton X-100 and then diluted with an equal volume of buffer A. The samples were centrifuged at

TABLE 2. Transfection of SR α -Wp85 into rat adipose cells^a

Group	SR α -Wp85 (μ g/cuvette)	SR α (μ g/cuvette)	GLUT4-HA (μ g/cuvette)
1	0	6	3
2	1	5	3
3	2	4	3
4	3	3	3
5	6	0	3
6	—	9	—

^a For each experiment, six groups of cells were transfected with various concentrations of SR α -Wp85. Cells from 10 cuvettes were pooled for each group. The amount and type of plasmid DNA used for each group are shown. Group 6 was transfected with SR α alone to determine the nonspecific signal. All cells were exposed to a total DNA concentration of 9 μ g per cuvette.

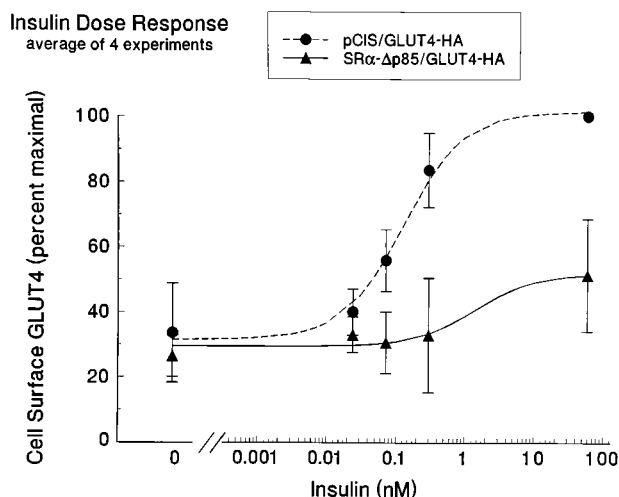


FIG. 1. Recruitment of epitope-tagged GLUT4 to the cell surface of isolated rat adipose cells cotransfected with either SRα-Δp85 and GLUT4-HA (▲) or pCIS2 and GLUT4-HA (●). The amount of each plasmid transfected is presented in Table 1. Results are the means \pm standard error of the mean (SEM) for four independent experiments. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (pCIS2/GLUT4-HA). The actual value for the specific cell-associated radioactivity for the control group at 60 nM insulin was 592 ± 63 cpm. The best-fit curve generated from the mean data for the control group had an ED_{50} of 0.13 nM. The difference in the two curves is statistically significant by multivariate analysis of variance ($F = 20.8$, $P = 0.00008$).

10,000 \times g for 5 min, and an aliquot of the supernatant from each sample containing 50 μ g of protein was brought to a final volume of 50 μ l with buffer A supplemented with 0.5% Triton X-100 and the protease inhibitor cocktail supplemented with soybean trypsin inhibitor (10 μ g/ml). The samples were incubated overnight on a rotating wheel at 4°C with 1 μ l of the anti-HA antibody 12CA5 (1 mg/ml) and 50 μ l of protein G-Sepharose (75% suspension washed with buffer A prior to use). The samples were pelleted by centrifugation at 2,500 \times g for 10 s and then washed twice at 4°C with 1 ml of buffer A with 0.1% Triton X-100, after which they were washed once with 50 mM HEPES, pH 7.4. The pellets were resuspended in 70 μ l of 20 mM Tris–10% sodium dodecyl sulfate (SDS)–10 mM dithiothreitol, pH 8.3, and incubated at room temperature with gentle agitation for 20 min. Then 7 μ l of 250 mM iodoacetic acid was added, and the incubation at room temperature was continued for an additional 20 min, after which 25 μ l of 4 \times Laemmli sample buffer was added. After 5 min, the samples were pelleted, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The contents of the gels were transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Immunological detection of GLUT4-HA was carried out with a rabbit polyclonal antiserum prepared against a 20-amino-acid peptide corresponding to the COOH-terminal sequence of GLUT4 (East Acres Biologicals) in conjunction with biotin-labeled goat anti-rabbit IgG (Chemicon International). The immunolabeled bands were visualized on film with a chemiluminescent detection system and quantitated by scanning densitometry.

Statistical analysis. Insulin dose-response curves were compared by a multivariate analysis of variance. Paired t tests were used to compare individual points when appropriate. P values of less than 0.05 were considered statistically significant. The insulin dose-response curves were fit to the equation $y = a + b [x/(x + k)]$ by using a Marquardt-Levenberg nonlinear least-squares algorithm. When plotted on linear-log axes, this equation gives a sigmoidal curve where the parameters are associated with the following properties: a is basal response, $a + b$ is maximal response, k is half-maximal dose (ED_{50}), and x is concentration of insulin.

RESULTS

Transfection of SRα-Δp85. To investigate the role of p85 in the insulin-stimulated translocation of GLUT4, we compared rat adipose cells cotransfected with a dominant negative mutant of p85 (SRα-Δp85) and epitope-tagged GLUT4 (GLUT4-HA) with cells cotransfected with pCIS2 (an empty expression vector) and GLUT4-HA (Fig. 1; see Table 1 for concentrations of DNA used). Total levels of GLUT4-HA, as determined by

immunoprecipitation of total membrane fractions with an anti-HA antibody followed by immunoblotting with an anti-GLUT4 antibody, were comparable for both groups of cells (Fig. 2). In control cells (pCIS2/GLUT4-HA), insulin caused an approximately threefold recruitment of epitope-tagged GLUT4 to the cell surface, with an ED_{50} of approximately 0.13 nM. In the absence of insulin, cell surface GLUT4 levels were not significantly different between control cells and transfected cells expressing Δp85. However, cells transfected with Δp85 (SRα-Δp85/GLUT4-HA) were markedly impaired in their ability to translocate epitope-tagged GLUT4 to the cell surface in response to insulin; that is, cell surface GLUT4 levels after insulin stimulation were not significantly different from basal levels. These results strongly suggest that functional p85 is required for the GLUT4 translocation response to insulin in adipose cells.

Transfection of SRα-Wp85. As another means of investigating the role of p85 in insulin-stimulated GLUT4 translocation, we overexpressed normal bovine p85 (Wp85) in rat adipose cells. When DNA concentrations of Wp85 that were the same as those in the Δp85 experiments (cf. Table 1) were used, we observed a significant increase in the basal cell surface GLUT4 level compared with that of control cells cotransfected with pCIS2/GLUT4-HA. However, the insulin dose-response curve of the cells transfected with Wp85 was relatively flat. It is possible that an excess of normal p85 relative to endogenous levels of p110 might interfere with signaling by competing with p85/p110 dimers for binding of phosphotyrosine residues on IRS-1. To investigate this issue, we tested the effect of transfecting different concentrations of SRα-Wp85 on insulin-stimulated translocation of GLUT4 (Fig. 3; see Table 2 for the DNA concentrations used). At lower levels of SRα-Wp85 (1, 2, and 3 μ g of DNA per cuvette), we did not observe any change in the basal GLUT4 levels compared with those of the control cells (cotransfected with SRα/GLUT4-HA). However, at the highest concentration of SRα-Wp85 tested (6 μ g of DNA per cuvette), we observed a statistically significant increase in the basal level of cell surface GLUT4, to a level that was approx-

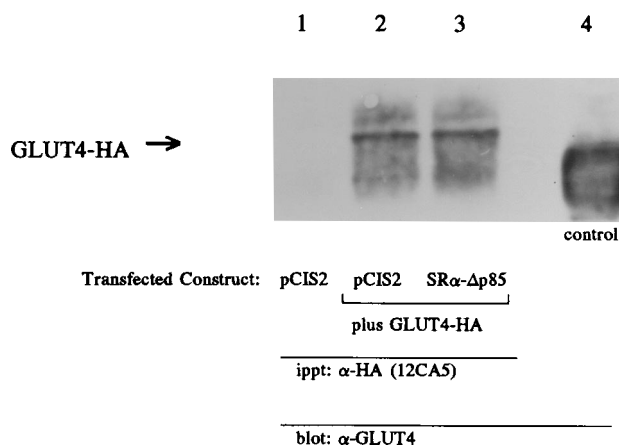


FIG. 2. Cotransfected cells express comparable levels of epitope-tagged GLUT4. Total membrane fractions from cells cotransfected with GLUT4-HA and either pCIS2 or SRα-Δp85 were immunoprecipitated (ippt) with the anti-HA antibody 12CA5 and immunoblotted with anti-GLUT4 antibody. Lane 1, sample from cells transfected with pCIS2 alone (negative control). Lane 4, extract of adipose cells that was used as a positive control for immunoblotting of GLUT4. The human epitope-tagged GLUT4 runs at a slightly higher position on the gel than the rat GLUT4. The intensities of the bands for the cells cotransfected with GLUT4-HA and pCIS2 or SRα-Δp85 are 9,100 and 9,600, respectively (in arbitrary density units).

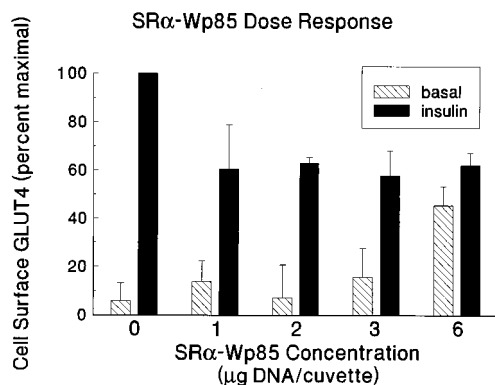


FIG. 3. Recruitment of epitope-tagged GLUT4 to the cell surface of isolated rat adipose cells cotransfected with GLUT4-HA and various concentrations of SR α -Wp85 and SR α . Each concentration of SR α -Wp85 was studied in the absence (hatched bars) or presence (solid bars) of 60 nM insulin. The total amount of plasmid DNA transfected was 9 μ g per cuvette for each condition (see Table 2). Results for the groups transfected with SR α -Wp85 at 0 and 6 μ g per cuvette are the means \pm SEM of nine independent experiments. Results for the remaining groups are the means \pm SEM of four independent experiments. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (SR α -Wp85 = 0). The actual value for the specific cell-associated radioactivity for the control group at 60 nM insulin was 798 ± 62 cpm. The basal cell surface GLUT4 (in the absence of insulin) for the cells transfected with 6 μ g of SR α -Wp85 per cuvette is significantly greater than the basal cell surface GLUT4 for the control group (SR α -Wp85 = 0). The cell surface GLUT4 levels for the cells transfected with 6 μ g of SR α -Wp85 per cuvette do not change significantly with insulin stimulation. The cell surface GLUT4 levels achieved with insulin stimulation are significantly lower in all groups than the GLUT4 levels seen with insulin stimulation of the control group (SR α -Wp85 = 0).

imately 50% of the cell surface GLUT4 seen with insulin stimulation of the control cells ($P = 0.004$). At all concentrations of SR α -Wp85 tested, the cell surface GLUT4 in the presence of 60 nM insulin was significantly less than the cell surface GLUT4 of the control cells in the presence of insulin. Nevertheless, a significant effect of insulin on GLUT4 translocation was observed at all DNA concentrations except the highest (6 μ g of DNA per cuvette).

Transfection of constitutively active ras mutants. To investigate the role of ras in insulin-stimulated translocation of GLUT4, we studied the effect of transfecting rat adipose cells with constitutively active mutants of ras (either pCIS-L61-ras or pCIS-v-ras) (Fig. 4 and 5; see Table 1 for concentrations of DNA used). Insulin stimulation of the control cells (cotransfected with pCIS2/GLUT4-HA) resulted in a ~ 3.3 -fold increase in the number of epitope-tagged GLUT4 at the cell surface, with an ED_{50} of approximately 0.1 nM. In the absence of insulin, the level of GLUT4 on the surface of cells expressing L61-ras was significantly higher than the basal level of GLUT4 on the surface of the control cells ($112\% \pm 20\%$ versus $28\% \pm 9\%$; $P = 0.014$, expressed as a percentage of cell surface GLUT4 seen with maximal insulin stimulation of the control cells). Insulin stimulation of cells expressing L61-ras resulted in a further statistically significant recruitment of GLUT4 to the cell surface ($141\% \pm 28\%$, $P = 0.02$).

Similar results were observed in cells transfected with pCIS-v-ras. That is, the basal level of GLUT4 on the surface of cells expressing v-ras was significantly higher than the basal level of GLUT4 on the surface of cells expressing v-ras was significantly higher than the basal level of GLUT4 on the surface of the control cells ($55\% \pm 14\%$ versus $33\% \pm 10\%$; $P = 0.018$). In addition, insulin stimulation of cells expressing v-ras resulted in a statistically significant increase in cell surface

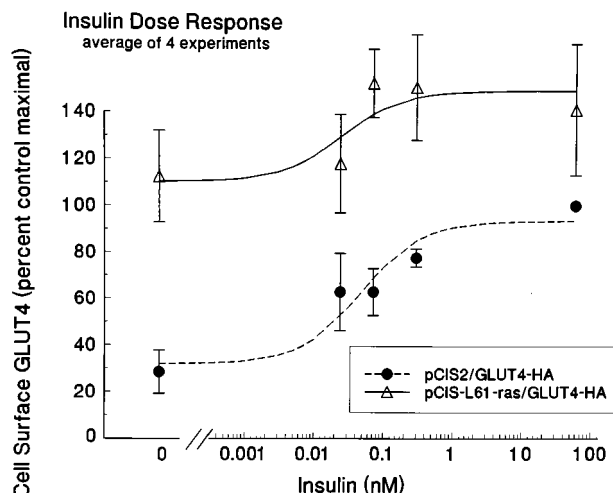


FIG. 4. Recruitment of epitope-tagged GLUT4 to the cell surface of isolated rat adipose cells cotransfected with either pCIS-L61-ras and GLUT4-HA (Δ) or pCIS2 and GLUT4-HA (\bullet). The amount of each plasmid transfected is presented in Table 1. Results are the means \pm SEM of four independent experiments. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (pCIS2/GLUT4-HA). The actual value for the specific cell-associated radioactivity for the control group at 60 nM insulin was 759 ± 136 cpm. The best-fit curve generated from the mean data for the control group had an ED_{50} of 0.05 nM. The difference in the two curves is statistically significant by multivariate analysis of variance ($F = 82$, $P = 3 \times 10^{-10}$).

GLUT4 ($150\% \pm 24\%$, $P = 0.03$). These data thus strongly suggest that activated ras is capable of stimulating the translocation of GLUT4 to the cell surface in adipose cells.

Transfection of a dominant negative ras mutant. As another means of investigating the role of ras in the insulin-stimulated translocation of GLUT4, we transfected adipose cells with a

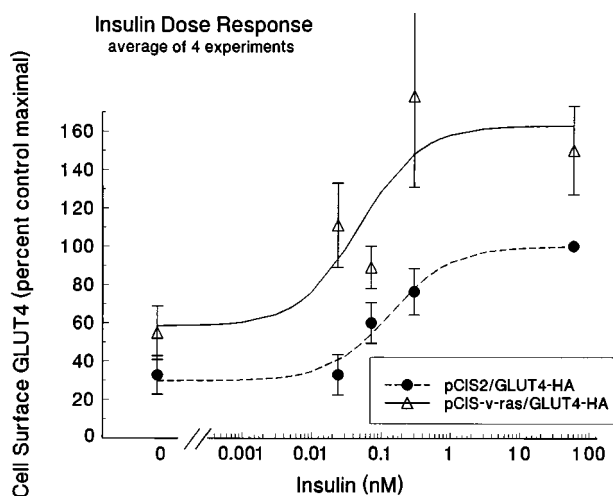


FIG. 5. Recruitment of epitope-tagged GLUT4 to the cell surface of isolated rat adipose cells cotransfected with either pCIS-v-ras and GLUT4-HA (Δ) or pCIS2 and GLUT4-HA (\bullet). The amount of each plasmid transfected is presented in Table 1. Results are the means \pm SEM of four independent experiments. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (pCIS2/GLUT4-HA). The actual value for the specific cell-associated radioactivity for the control group at 60 nM insulin was 600 ± 104 cpm. The best-fit curve generated from the mean data for the control group had an ED_{50} of 0.13 nM. The difference in the two curves is statistically significant by multivariate analysis of variance ($F = 18$, $P = 0.0002$).

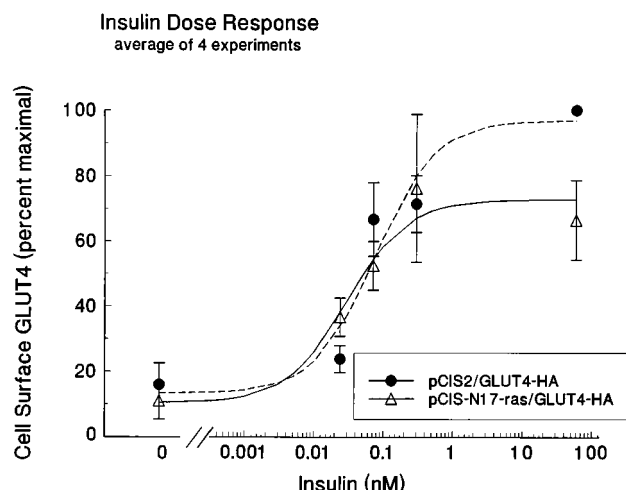


FIG. 6. Recruitment of epitope-tagged GLUT4 to the cell surface of isolated rat adipose cells cotransfected with either pCIS-N17-ras and GLUT4-HA (Δ) or pCIS2 and GLUT4-HA (\bullet). The amount of each plasmid transfected is presented in Table 1. Results are the means \pm SEM of four independent experiments. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (pCIS2/GLUT4-HA). The actual value for the specific cell-associated radioactivity for the control group at 60 nM insulin was 772 ± 131 cpm. The best-fit curve generated from the mean data for the control group had an ED_{50} of 0.08 nM. There is no significant difference between the two dose-response curves when analyzed by multivariate analysis of variance ($F = 1.8$, $P = 0.2$).

dominant negative mutant of ras. In contrast to cells expressing constitutively active ras mutants, cells expressing N17-ras had an insulin dose-response curve that was not significantly different from that observed with the control cells (Fig. 6). At the 60 nM insulin dose, the amount of GLUT4 recruited to the cell surface in cells expressing N17-ras was slightly lower than that of the control cells. However, this difference was not statistically significant when analyzed by the paired t test ($P = 0.14$). Thus, inhibition of ras function does not appear to affect insulin-stimulated recruitment of GLUT4 in adipose cells.

Immunoprecipitation and immunoblotting of epitope-tagged GLUT4 in cotransfected cells. To determine if transfection of the various ras constructs altered the level of expression of cotransfected epitope-tagged GLUT4, we immunoprecipitated GLUT4-HA from total membrane fractions of cotransfected cells with an antibody directed against the HA epitope and then immunoblotted with an anti-GLUT4 antibody (Fig. 7). The total amount of GLUT4-HA detected by this method was comparable in all groups of cells. Therefore, the large increase in GLUT4-HA detected at the cell surface of cells cotransfected with either L61-ras or v-ras is probably caused by recruitment of GLUT4 from an intracellular pool and not related to alterations of the total amount of GLUT4-HA expressed in the various groups of cotransfected cells.

Cotransfection of ras constructs and SRE-luc. To verify that the ras mutants were functioning as expected, we cotransfected the various ras constructs with the serum response element of *c-fos* driving a luciferase reporter gene (SRE-luc) and measured the effect of the ras mutants to modulate insulin-stimulated luciferase activity (Table 3). Insulin increased the luciferase activity by approximately 50% in cells transfected with the SRE-luc plasmid ($P = 0.02$). Cells cotransfected with pCIS-L61-ras showed a large increase in luciferase activity (in the absence of insulin) to levels approximately four times that of the control cells stimulated with insulin. Cells transfected with pCIS-v-ras also demonstrated a statistically significant

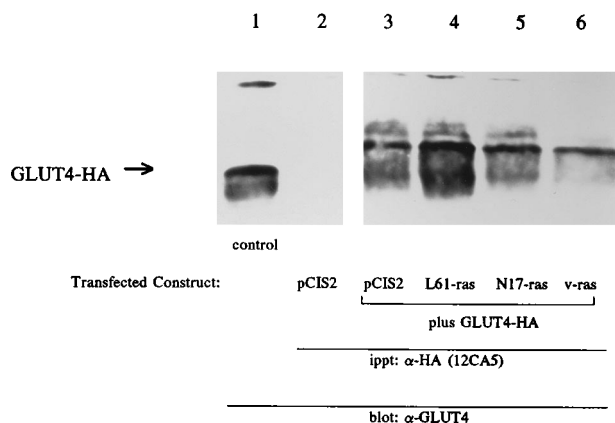


FIG. 7. Cotransfected cells express comparable levels of epitope-tagged GLUT4. Total membrane fractions from cells cotransfected with GLUT4-HA and either pCIS2, pCIS-L61, pCIS-v-ras, or pCIS-N17-ras were immunoprecipitated (ippt) with the anti-HA antibody 12CA5 and immunoblotted with anti-GLUT4 antibody. Lane 1, extract of rat skeletal muscle that was used as a positive control for immunoblotting of GLUT4. Lane 2, sample from cells transfected with pCIS2 alone (negative control). The human epitope-tagged GLUT4 runs at a slightly higher position on the gel than the rat GLUT4. The intensities of the bands (in arbitrary units) are 13,600, 18,400, 11,500, and 9,700 for lanes 3 to 6, respectively. This is a representative blot from experiments that were repeated independently three times.

increase in luciferase activity over the control cells, although the effect was not as large as for pCIS-L61-ras. Thus, the ability of these two mutant forms of ras to induce expression of SRE-luc is consistent with the fact that both L61-ras and v-ras are constitutively active. Expression of dominant negative N17-ras led to an approximately 30% decrease in luciferase activity in cells incubated in the presence or absence of insulin. However, the inhibition due to N17-ras was statistically significant only in the presence of insulin ($P = 0.001$).

Effect of wortmannin on adipose cells transfected with ras mutants. Wortmannin is an inhibitor of PI 3-kinase that abolishes the effect of insulin to stimulate recruitment of GLUT4 and glucose transport in adipose cells and 3T3-L1 adipocytes (4, 23). Therefore, we compared insulin-stimulated recruitment of epitope-tagged GLUT4 in transfected cells incubated in the presence and absence of 500 nM wortmannin to determine if wortmannin could block the ability of the constitutively active ras mutants to recruit GLUT4 to the cell surface (Fig.

TABLE 3. Cotransfection of rat adipose cells with SRE-luc and ras constructs^a

Group	Mean relative luminescence (%) \pm SEM	
	Basal	Insulin
pCIS2	71 \pm 8	100
pCIS-L61-ras	385 \pm 115	439 \pm 80
pCIS-v-ras	106 \pm 5	163 \pm 29
pCIS-N17-ras	54 \pm 5	71 \pm 3

^a Cells from 12 cuvettes were pooled for each group. A control group was cotransfected with SRE-luc and pCIS2. SRE-luc was used at 1 μ g per cuvette, and ras constructs were used at 6 μ g per cuvette. Electroporated cells were incubated overnight and then treated or not with 60 nM insulin for 6 h. Results are the means of four independent experiments, expressed as a percentage of the luminescence of the control group after insulin stimulation. Insulin caused a statistically significant stimulation of luciferase activity only in cells transfected with pCIS2 ($P = 0.02$). The basal luciferase activity of cells transfected with L61-ras or v-ras was significantly higher than the basal activity of cells transfected with pCIS2 ($P = 0.04$). Insulin-stimulated luciferase activity in N17-ras-transfected cells was significantly lower than in pCIS2-transfected cells ($P = 0.001$).

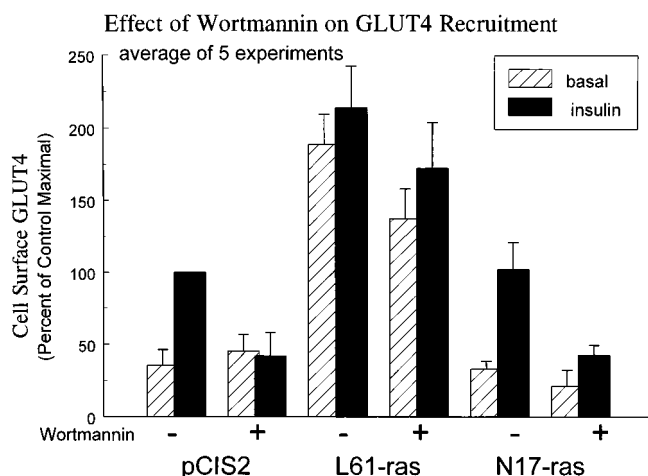


FIG. 8. Effect of wortmannin on rat adipose cells transfected with ras mutants. After electroporation, cells were treated or not with wortmannin (500 nM) prior to treatment with or without insulin (60 nM) for 30 min. Since incubation of cells with wortmannin for either 20 h or 30 min before insulin treatment gave similar results, the results of these experiments were combined. Results are the means \pm SEM of five independent experiments. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (pCIS2/GLUT4-HA in the absence of wortmannin). The actual value for the specific cell-associated radioactivity for the control group at 60 nM insulin was 929 ± 66 cpm. Insulin stimulation caused a significant increase in levels of cell surface GLUT4 over the basal levels only for cells transfected with either pCIS2 or N17-ras and incubated without wortmannin ($P = 0.002$ and 0.01 , respectively). In the absence of insulin, the basal levels of cell surface GLUT4 were comparable for all groups except for the L61-ras group, which had basal levels significantly higher than the control group. Cells expressing L61-ras in the presence of wortmannin and absence of insulin had levels of cell surface GLUT4 that were comparable to those of the control group stimulated with insulin. The other groups of cells expressing L61-ras all had significantly higher levels of cell surface GLUT4 than the control group stimulated with insulin. Wortmannin treatment did not significantly change the basal level of cell surface GLUT4 within any of the groups except for the cells expressing L61-ras, for which there was a significant decrease ($P = 0.0004$).

8). As expected, wortmannin treatment of the control cells (cotransfected with pCIS2 and GLUT4-HA) abolished insulin-stimulated recruitment of GLUT4. In contrast, for cells overexpressing L61-ras, wortmannin caused only a 25% decrease in the significantly elevated level of cell surface GLUT4 seen in the absence of insulin. In the presence of insulin, wortmannin did not significantly affect the level of GLUT4 at the surface of cells overexpressing L61-ras. Insulin-stimulated recruitment of GLUT4 in cells expressing the dominant negative N17-ras was abolished by wortmannin treatment. Thus, the recruitment of GLUT4 in response to constitutively active ras is not absolutely dependent on the signaling pathways that are blocked by wortmannin.

DISCUSSION

PI 3-kinase catalyzes the phosphorylation of phosphatidylinositol (PI), PI(4)P, and PI(4,5)P₂ at the D3 position of the inositol ring, resulting in PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃ (24). The cellular accumulation of these phosphorylated PI derivatives has been linked with growth factor signaling through ligand-activated receptor tyrosine kinases, mitogenesis, and cellular transformation in many different contexts (24, 43). However, the precise role of these potential lipid second messengers in signaling remains unknown. Recently, a putative role for PI 3-kinase in the metabolic signaling pathway of insulin has been suggested by the fact that inhibitors of PI 3-kinase, such as wortmannin and LY294002, can abolish in-

sulin-stimulated glucose transport in rat adipose cells (23) and 3T3-L1 adipocytes (3, 6). However, interpretation of these studies is complicated by the possibility that these inhibitory compounds exert unrecognized effects that are not specific to PI 3-kinase activity.

Transfection of SR α - Δ p85. In CHO cells stably transfected with insulin receptors and Δ p85, it has previously been shown that PI 3-kinase activity is important for the stimulation of glucose transport by insulin (mediated primarily by GLUT1 translocation in these tissue culture cells) (9). Δ p85 retains the ability to bind to phosphorylated YMXM motifs but lacks the ability to bind the p110 catalytic subunit of PI 3-kinase. Thus, in transfected CHO cells, Δ p85 disrupted the formation of signaling complexes by blocking the binding of functional p85/p110 to phosphorylated IRS-1. However, other insulin-signaling pathways, such as phorbol myristate acetate stimulation of glucose transport and insulin-stimulated activation of ras, remained intact.

We have extended these observations by transiently expressing Δ p85 in the physiologically relevant rat adipose cell. The striking ability of Δ p85 to abolish insulin-stimulated translocation of GLUT4 in transfected adipose cells strongly suggests an essential role for p85 in insulin-stimulated glucose transport in insulin target tissues. Although it is possible that p85 may be interacting with other signaling proteins besides p110 (39), it seems likely that PI 3-kinase activity resulting from the activation of p110 by p85 is somehow involved in insulin-stimulated glucose transport mediated by GLUT4 translocation. Thus, our data demonstrating that p85 is important for insulin-stimulated translocation of GLUT4 provide direct evidence in physiologically relevant cells to support the conclusion that PI 3-kinase is essential to mediating the effect of insulin to recruit GLUT4 to the cell surface.

One of the limitations of our adipose cell transfection system is the small fraction of cells transfected ($\sim 5\%$). Therefore, it was not possible to directly assess the effects of transfected Δ p85 on the formation of signaling complexes or PI 3-kinase activity as was done in the studies with stably transfected CHO cells (9). However, using an epitope-tagged GLUT4 as a reporter for GLUT4 translocation, we were able to monitor GLUT4 translocation exclusively in the fraction of cells that are transfected. Our demonstration of a dramatic reduction in insulin-stimulated translocation of epitope-tagged GLUT4 caused by cotransfection of Δ p85 is good presumptive evidence that the dominant negative mutant was being expressed in sufficient quantity to have a significant functional effect.

Transfection of SR α -Wp85. One would predict that the effects of overexpression of p85 would depend upon the availability of excess free p110 catalytic subunit to complex with p85. If sufficient quantities of free p110 were available, then expression of additional p85 might stimulate GLUT4 translocation. In contrast, in the absence of free p110, overexpression of p85 might exert a dominant negative effect to inhibit insulin action. In fact, we observed both agonistic and antagonistic effects of overexpression of p85. Transfection of high levels of Wp85 results in a significant increase in the basal level of cell surface GLUT4 (in the absence of insulin). This suggests that a small signal present in the basal state can be amplified by an excess of p85. This interpretation is consistent with our previous observations that overexpression of either human insulin receptors or human IRS-1 in rat adipose cells is sufficient to significantly increase cell surface GLUT4 levels in the absence of insulin (30, 31). Our interpretation assumes the availability of free p110 to associate with the excess of Wp85. However, expression of low levels of Wp85 caused a significant decrease in the amount of GLUT4 that could be recruited to the cell

surface in response to insulin. Perhaps overexpression of one subunit of PI 3-kinase decreases the ability to form active signaling complexes of IRS-1/p85/p110 in response to insulin. That is, the pool of active complexes is diluted by an excess of one of the components. This interpretation is consistent with other studies reporting that overexpression of p85 interferes with the ability of insulin to stimulate the serum response element of the *c-fos* promoter (48).

The fact that we see inhibition of insulin signaling at low levels of Wp85 but amplification of the basal signal only at high levels of Wp85 is difficult to explain. One possibility is that Wp85 interacts with other signaling pathways that modulate GLUT4 translocation. The principal insulin signaling pathway leading to PI 3-kinase activation is thought to involve the binding of SH2 domains on p85 to YXXM motifs on IRS-1 that have been phosphorylated by the insulin receptor tyrosine kinase (1, 5, 22, 50). In addition, direct interaction between a phosphorylated YXXM motif in the COOH terminus of the insulin receptor and the SH2 domains of p85 results in activation of PI 3-kinase *in vitro* (16, 45). Interestingly, IRS-1 may be located both upstream and downstream of PI 3-kinase, since IRS-1 appears to be a substrate for the serine kinase activity of PI 3-kinase (15). In addition to containing SH2 domains, p85 also contains an SH3 domain and a proline-rich sequence which can bind to SH3 domains. Recently, it has been shown that activation of PI 3-kinase can occur through the interaction of SH3 domains of src family kinases and the proline-rich sequence of p85 (27). It is also conceivable that p85 may aggregate with itself through interactions of the SH3 domain on one p85 molecule with the proline-rich sequence of another p85 molecule. In addition to ligand-activated receptor tyrosine kinases, other upstream activators of PI 3-kinase activity include the small GTP-binding protein Rho (51) and the heterotrimeric G proteins (41, 42). Thus, it is not straightforward to interpret experiments in which Wp85 is overexpressed.

Role of ras in insulin-stimulated GLUT4 translocation. Insulin activation of ras occurs through a signal transduction pathway that involves the insulin receptor tyrosine kinase's phosphorylating cellular substrates such as Shc and IRS-1 (21, 28, 34, 49). These tyrosine-phosphorylated substrates bind specifically to the SH2 domain of the adaptor molecule GRB-2, which is preassociated with the guanine nucleotide exchange factor Sos. When Sos is recruited to the insulin signaling complex by GRB-2, it promotes the formation of ras in the active GTP-bound state (37). While an important role for ras in the mitogenic actions of insulin is well established (12, 18, 20, 34), there are conflicting views in the literature regarding the importance of ras in the metabolic actions of insulin (2, 10, 14, 19, 44). Our experiments on the role of ras in GLUT4 translocation are the first such studies in a bona fide insulin target tissue.

Constitutively active ras recruits GLUT4. In CHO cells overexpressing insulin receptors, it has previously been shown that insulin stimulation of a luciferase reporter gene under the control of the *c-fos* serum response element (SRE-luc) is dependent, in part, on ras signaling (48). To verify that L61-ras and v-ras function in a constitutively active manner under our experimental conditions, we cotransfected pCIS-L61-ras or pCIS-v-ras with SRE-luc. As expected, we observed an increase in luciferase activity in transfected adipose cells expressing the constitutively active ras mutants (L61-ras > v-ras > control). Thus, the two mutant forms of ras are expressed and function in a constitutively active manner in our experimental system. Since the total amount of GLUT4-HA expressed in the cotransfected cells is not substantially altered by the presence of the ras constructs (as determined by immunoprecipitation and immunoblotting), changes in cell surface GLUT4-HA are

presumably related to changes in the translocation process. The fact that overexpression of L61-ras or v-ras in rat adipose cells results in an increase in the amount of cell surface GLUT4, even in the absence of insulin, suggests that activated ras is sufficient to recruit GLUT4. In the absence of insulin, L61-ras had a bigger effect on GLUT4 recruitment than v-ras, consistent with both the results of the SRE-luc cotransfection experiments and what has previously been observed regarding the relative potencies of these mutants (17). Insulin stimulation of cells transfected with the constitutively active ras mutants resulted in a further increase in the level of cell surface GLUT4. This suggests that signaling pathways used by activated ras are at least partially independent of insulin signaling pathways leading to GLUT4 recruitment.

Our results with the constitutively active ras mutants are consistent with data reported by Kozma et al., who found that 3T3-L1 adipocytes stably transfected with a constitutively active mutant of ras (K61-ras^N) had the majority of GLUT4 located in the plasma membrane (14). However, they did not observe recruitment of additional GLUT4 to the cell surface with insulin stimulation. Differences in cell type as well as the fact that stably transfected 3T3-L1 cells are chronically exposed to activated ras for a longer period than transiently transfected adipose cells may explain this minor discrepancy between the results obtained in the two laboratories. Our results do not agree with data reported by Hausdorff et al., who found that microinjection of 3T3-L1 adipocytes with a constitutively active ras mutant (V12-ras) did not affect the acute insulin stimulation of GLUT4 translocation (10). It is possible that the constitutively active ras mutant used by Hausdorff et al. is not as potent as the mutants that we used. In addition, microinjected 3T3-L1 tissue culture cells may not behave identically to rat adipose cells.

N17-ras has no effect on GLUT4 recruitment. When we cotransfected N17-ras with SRE-luc, we observed an inhibition of insulin-stimulated luciferase activity, suggesting that under our experimental conditions, N17-ras is being expressed and behaving in a dominant negative fashion. However, transfection of dominant negative ras did not significantly alter the ability of insulin to recruit GLUT4 in transfected rat adipose cells. This suggests that insulin-stimulated GLUT4 recruitment in adipose cells is independent of ras signaling. These results are consistent with those of Hausdorff et al., who found that microinjection of 3T3-L1 cells with either a dominant negative ras mutant or neutralizing antibodies directed against ras had no effect on insulin-stimulated GLUT4 recruitment (10).

Evidence that ras signaling of GLUT4 recruitment is independent of PI 3-kinase. Our experiments with Δ p85 as well as recent studies with inhibitors of PI 3-kinase, such as wortmannin and LY294002 (3, 6, 23), suggest that insulin signaling through PI 3-kinase is essential for insulin-stimulated GLUT4 translocation and glucose transport. We incubated transfected adipose cells with wortmannin to determine if activated ras can recruit GLUT4 independently of insulin signaling. Although wortmannin treatment (either overnight or 30 min before insulin stimulation) completely blocked the insulin-stimulated translocation of GLUT4 in the control cells, wortmannin inhibited the effect of L61-ras to recruit GLUT4 to the cell surface by only 25%. Recently, it has become apparent that some isoforms of PI 3-kinase exist that may not be inhibited by wortmannin (11, 38). Therefore, it remains possible that activated ras may be recruiting GLUT4 by stimulating an isoform of PI 3-kinase that is not inhibited by wortmannin. Although PI 3-kinase has been implicated both upstream and downstream from ras (33, 36, 48), the simplest interpretation of our data

suggests that recruitment of GLUT4 by activated ras in transfected adipose cells is likely to be independent of PI 3-kinase.

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